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Phage-based target discovery and its exploitation towards novel antibacterial molecules

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The deeply intertwined evolutionary history between bacteriophages and bacteria has endowed phages with highly specific mechanisms to hijack bacterial cell metabolism for their propagation. Here, we present a comprehensive, phage-driven strategy to reveal novel antibacterial targets by the exploitation of phage-bacteria interactions. This strategy will enable the design of small molecules, which mimic the inhibitory phage proteins, and allow the subsequent hit-to-lead development of these antimicrobial compounds. This proposed small molecule approach is distinct from phage therapy and phage enzyme-based antimicrobials and may produce a more sustainable generation of new antibiotics that exploit novel bacterial targets and act in a pathogen-specific manner.

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The current limitations of antibiotic development threaten our long-term capacity to treat bacterial infections

Since their discovery in 1928 and development for clinical use in the 1940s, antibiotics have saved millions of lives and are deeply ingrained in countless important medical procedures. Unfortunately, antibacterial drug resistance among clinically important pathogens is increasing to dangerous levels worldwide and jeopardize the efficacy of clinical treatments [1]. Over the past twenty years, only a few new classes of antibiotics have been discovered and approved for clinical trials [2]. Primarily, small and medium-sized enterprises carry out antibacterial development, often using a traditional R&D strategy based on chemical modification of existing molecules [3]. The current clinical development pipelines for antibiotics mainly focus on the combinations of known antibiotics and that remain as the principal to limit the development of antibiotic resistance [4]. Therefore, only a limited number of antibacterial targets are being exploited. As of July 2020, among 45 new antibiotics in the clinical development only eleven belong to a novel class, targeting primarily Gram-positive bacteria (The Pew Charitable Trusts; URL: https://www.pewtrusts.org/en/research-and-analysis/data-visualizations/2014/antibiotics-currently-in-clinical-development). Most antibiotics have broad spectra of activity against various bacterial species, not only exacerbating the development of resistance if used inappropriately [5], but also having a negative impact on commensal bacteria, for example, the gut microbiota [6].

To date, the number of targets exploited by commercial antibacterial agents is estimated around 40 [3]. However, with the development of 'omics' technologies, numerous novel modes of action and an unprecedented number of bacterial cell structures could potentially be exploited, even using uncharacterised antimicrobial agents [7•]. Therefore, there is an urgent need to accelerate the development of new antimicrobial agents and to identify novel antibacterial targets that are less prone to resistance development and act in a more specific way. In this review we explore the untapped potential of bacteriophages in this area with an expanded strategy for small-molecule development.

The interaction between bacteriophages and bacteria driven by their deeply entangled co-evolutionary histories

Throughout evolution, bacteriophages continuously adapt to confront their bacterial hosts in an arms-race during the lytic infection cycle. Phages use sophisticated molecular mechanisms to hijack the bacterial cell metabolism for the production of progeny viral particles [8,9]. This closely intertwined relationship has led to the emergence of highly specific and evolution-optimised molecular interactions [10•,11] and therefore represents a

unique source to identify new potential antibacterial targets.

Following the advancements in high-throughput genome sequencing, tens of thousands of phage genomes have been sequenced. Ranging from 2.3 kb to 735 kb in genome size [12,13], phages encode precise information to alter DNA replication, RNA transcription, cell division, and protein translation pathways in the host bacteria [10•]. However, due to the absence of homology to known proteins and the lagging in experimental evidence, approximately half to two thirds of the phage gene products have merely been annotated as hypothetical proteins of unknown function [14]. Most of these hypothetical proteins are expressed during the early stages of phage infection [15–17], which indicates their importance in host metabolism takeover. Looking into the functions of these hypothetical phage proteins, especially those with detrimental effect towards host growth, would not only reveal detailed phage biology insights but, perhaps more importantly, also understand bacterial reprogramming during phage infection.

Phage-inspired antibacterial target discovery

Revealing new bacterial targets from phage researches can inspire the screen and design of new small molecular compounds, which mimic the growth-inhibitory effects of the phage proteins. A key example of phage-derived antibacterial discovery was first described in 2004. From the genomes of 26 *Staphylococcus aureus* phages, Liu *et al.* [18] found that 31 polypeptide families showed toxicity towards the host when expressed in *S. aureus*. One of such gene products, ORF104 from *S. aureus* phage 77, interacts with helicase loader (DnaI), an essential protein in host DNA replication. As a novel target, the interaction between DnaI and ORF104 was subsequently used in time-resolved fluorescence energy transfer (TR-FRET) to screen for small molecules inhibitors. In the end, 36 out of 125 000 screened commercial small molecules could interrupt the molecular interaction between DnaI and ORF104, and 11 were directly active against *S. aureus* at reasonable MIC ($\leq$16 μg/ml).

Expanding phage-inspired target discovery by informed functional insights

Although the approach proposed by Liu *et al.* [18] was focused on *S. aureus* and set for blind screening of phage proteins and subsequent small molecules, its principle has inspired researchers to look for novel molecular targets from phages [19•]. Beyond Gram-positive *S. aureus*, this screening technique has been applied to *Rhodococcus equi* [20], *Mycobacterium smegmatis* [21], and Gram-negative pathogens including *Escherichia coli* [22], *Pseudomonas aeruginosa* [23–25], *Yersinia enterocolitica* [26] and *Klebsiella pneumoniae* [27•]. In our own research, we aim to expand this original phage-based target discovery strategy to identify and to exploit novel antibacterial targets for drug discovery in a more comprehensive manner. This strategy is driven by two complementary approaches: the 'phage genome-driven screening' which starts from known toxic phage proteins, leading to target discovery, and a 'bacterial target-driven screening' to identify phage-encoded inhibitors against known presumptive bacterial targets. Following confirmation of these interactions, these approaches converge towards the design or screen of small mimicking molecules for preclinical development (Figure 1). These strategies are outlined and illustrated in the next sections.

Bacteriophage genome-driven screening

Because of the abundance of hypothetical proteins in phage genome annotations, it is rational to search for bactericidal polypeptides with novel targets among the 'early' hypothetical proteins of unknown function. Advances in DNA sequencing technologies, protein structure determination, and interaction modelling have facilitated the screening process, and several systematic methodologies have been developed. In this section, we compare genomic screenings inspired by the study of Liu *et al.* [18] and explore more optimised approaches for target discovery.

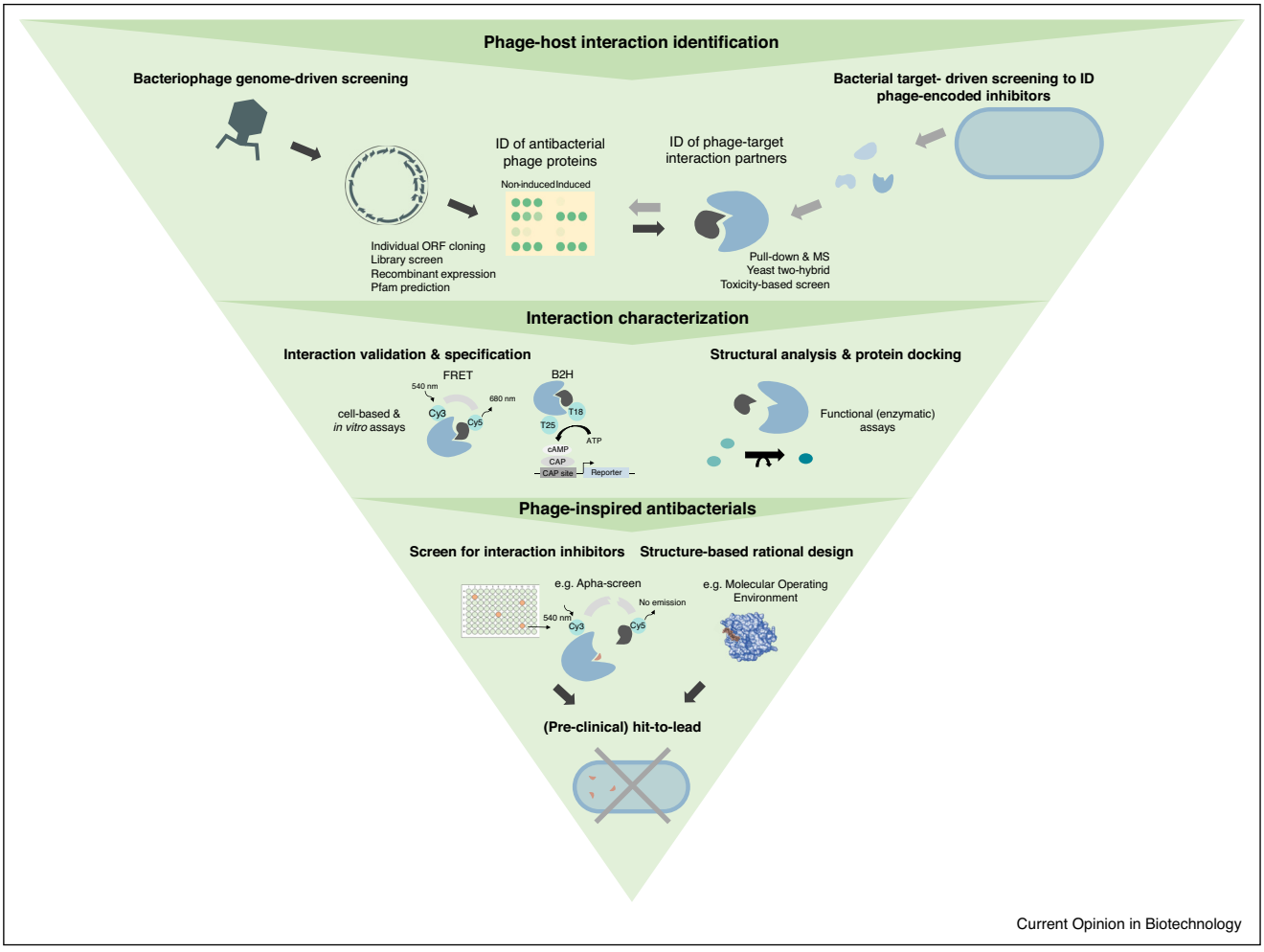
Selecting phage material for genomic screening

Screening individual hypothetical genes from phage genomes is a time consuming and laborious process. Therefore, thoughtful selection of the test pool can reduce efforts and maximise the efficiency to identify antibacterial phage proteins. Apart from eliminating the genes encoding proteins of known function (including structural proteins) and proteins with predicted transmembrane domains, focusing on small and early genes has significantly reduced the work load in the screening of phage genes [23,24,26]. Indeed, most reported phage-host interactions occur at early stage upon infection and involve small ($\leq$250 amino acids) phage proteins [28]. Alternatively, libraries with random phage genome fragments instead of individual genes can be used, skipping the labour-intensive step of individual gene cloning. However, an additional step is required to identify the specific gene in the fragment that attenuates the bacterial growth [20,21]. In this way, besides specific phage proteins, unpredicted bactericidal polypeptides can be identified [21].

Identifying antibacterial phage proteins

To detect growth inhibition caused by hypothetical phage proteins, high throughput methods are desirable to shorten the screening process. As such, we recently developed a screening method based on high throughput sequencing. After eliminating phage-particle associated proteins with LC–MS/MS, all 'true' hypothetical phage gene fragments were ligated to a high-copy vector pU11L4 for transformation and sequencing using Illumina HiSeq. Since 'toxic' gene products do not result in

Figure 1

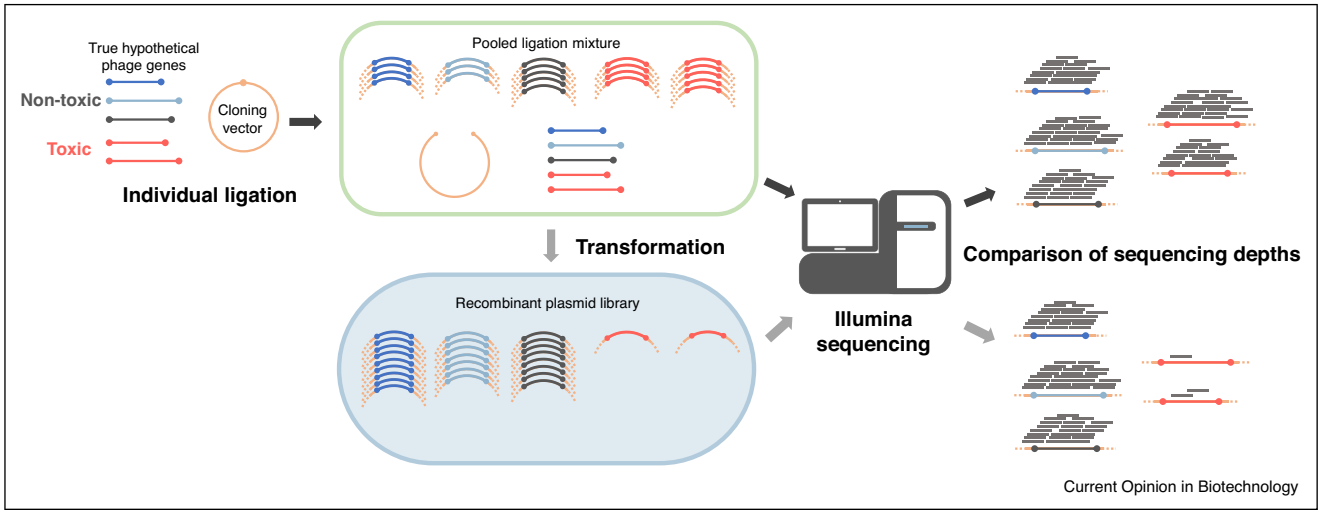


Phage-based target discovery and its exploitation in the development of novel antibacterials. Two complementary yet integrated approaches are proposed: 1) 'phage genome-driven screening' searches for antibacterial gene products within sequenced bacteriophage genomes. These bactericidal phage proteins are used as baits to identify their interaction partners in bacterial cells. 2) 'target-driven screening' uses promising essential bacterial protein complexes as targets to identify interacting phage proteins which inhibit the target. In a next step, these phage-host interactions are characterised structurally and cross-validated. Finally, either a high-throughput screening assay can be developed to identify antimicrobial small molecules against the phage-bacterial targets, or a structure-based approach can be applied up to rationally design phage peptide-mimicking molecules. The resulting small molecules should also be tested for their antibacterial properties, cytotoxicity, solubility and additional pharmacodynamic and pharmacokinetic properties as potential leads. ORF, open reading frame; ID, identification/identify; MS, mass spectrometry; FRET, Förster/fluorescence resonance energy transfer; B2H, bacterial two-hybrid; Alpha-screen, amplified luminescent proximity homogenous assay screen.

viable recombinant plasmids for the cell, a negative selection based on reduced sequencing depth for these toxic loci is possible. Indeed, bactericidal polypeptides can be pinpointed from these regions with low sequence coverage depth (Figure 2, Kasurinen J, unpublished).

Although high-copy expression can efficiently identify antibacterial phage proteins, false-positive results may emerge due to overexpression-associated growth retardation. To avoid this problem, an integrative system based on pUC18-mini-Tn7T-Lac vector compatible to gateway cloning system was applied to obtain single-copy, inducible expression of hypothetical phage gene, optimized with translational enhancers [23,24,29•]. The inhibitory effect caused by phage proteins can be identified using either phage's natural host or alternative bacterium such as *E. coli*. The advantage of testing in *E. coli* roots in its extensively available genetic tools, and is based on the assumption that the target molecule is prevalent among closely related bacteria [26,27•]. Yet, since narrow-spectrum targets are often preferred for future antimicrobial treatment [2,4,30], the identified antibacterial phage

Figure 2



Screening scheme based on high throughput sequencing to identify antibacterial phage proteins.

proteins should be tested in multiple hosts to determine the range of toxicity and conservation of the bacterial target [21,23,24,31].

Identifying bacterial targets of antibacterial phage proteins

Once antibacterial phage proteins are identified, they can serve as a bait to discover antibacterial targets. This identification of the bacterial interaction partner remains a key aspect. For this, different techniques are available, including pull-down followed by mass spectrometry [18,31], yeast two-hybrid [24] and genome-based screens that focus on abolishing the toxicity by either deleting [22] or overexpressing [32] the target protein. Suitable new targets for drug discovery should be absent in eukaryotes, conserved in (specific) bacteria, preferentially essential for growth, and susceptible to the inhibition by antibacterials. Moreover, the mechanism of inhibition must be distinct from existing antibiotics to avoid cross-resistance [33]. To date, the most promising protein candidates include those impacting host replication (the **g**ene **p**roduct Gp8 of phage N4 against *E. coli* DNA polymerase HolA), transcription (Gp2 from *E. coli* phage T7 against RNA polymerase RpoD), and cell division machineries (T7's Gp0.4 targeting the cell division protein FtsZ) [19•]. However, phage-host studies reveal that much more key processes in the host are targeted by phages [10•].

Bacterial target-driven screening to identify phage-encoded inhibitors

Unlike the phage genome-driven screening approach where the screening process begins with massive genome mining, target-driven screening uses defined essential protein complexes in the host cells as baits to fish out unknown interaction partners from bacteriophages. Meanwhile, this approach leads to the discovery and analysis of mechanisms that govern these key bacterial processes. In an early study by Van den Bossche *et al.* [25], essential protein complexes involved in *Pseudomonas* metabolism were selected as baits, including RpoA, DnaN for transcription and genome replication, MvaT, Hfq for transcriptional regulation, FtsZ for cell division, AcpP for fatty acid biosynthesis, and GlcB for energy maintenance. Mutant strains containing these tagged protein complexes are infected by phage and subjected to pull-down and downstream mass spectrometry. In this analysis, 37 phage interaction partners were identified, eight of which directly affected the bacterial growth [25]. Using a similar method, Klimuk *et al.* [34] identified RNA polymerase subunit β' (RpoC) interacting proteins, Gp25.1 and Gp36 of *Pseudomonas* phages LUZ19 and LKA1, respectively. Their functional equivalent in *E. coli* phage T7 is Gp2, which stand-alone can inhibit the function of host RNA polymerase [35]. Moreover, Dip/Gp37 of *Pseudomonas* phage phiKZ, an RNA degradosome interacting protein, was found to bind the RNA binding sites on the RNaseE component to reduce RNA turnover [36••]. Also, an inhibitor of transcriptional regulator MvaT was identified from *P. aeruginosa* phage LUZ24, Mip/Gp4, which prevents MvaT from binding to foreign DNA and hence ensures the viral transcription [24].

Mimicking the growth-inhibitory effect of phage proteins

Bacterial proteins that are selectively inhibited by phage polypeptides could potentially serve as novel antibacterial targets, as their disruption could destabilise essential processes and lead to growth retardation of the bacterium.

Further characterisation of the antibacterial mechanism, as well as specification of the interaction site should be performed to establish an efficient method to identify small molecules or antimicrobial peptides that mimic the antibacterial effect of the phage proteins [18,37•]. Moreover, the experimental approach should be combined with structural and biophysical analysis and interaction modelling to enable a direct design of potent inhibitors against the identified targets [38]. Structures of a handful phage proteins targeting promising antibacterial targets have been determined by high-resolution X-ray crystallography [37•,39], nuclear magnetic resonance (NMR) [35,40] and cryo-electron microscopy (cryo-EM) [41,42•]. For example, two distinct proteins, Gam from *E. coli* phage λ and Orc/Gp0.3 from T7, were structurally characterised via cryo-EM. Both were found mimicking DNA and might be exploited for therapeutic inhibitors of DNA binding proteins [41,42•]. Similarly, via X-ray crystallography, the Dip protein of phage phiKZ was found to bind the RNA binding site [36••], which may inspire the discovery of therapeutic inhibitors of RNA turnover [43,44]. Using NMR, Gp2 from *E. coli* phage T7 was shown to alter the conformation of RNA polymerase, thus restricting the access of ssDNA to the active site. This inactivation represents an alternative mechanism to deactivate bacterial RNA polymerase [35,45]. In addition, Hood and Berger [37•] further structurally and biochemically characterised the interaction between ORF104 of *S. aureus* phage 77 and DnaI, the most promising interaction from the initial screen by Liu et al. in 2004 [18], and localised the interaction site to a small hydrophobic pocket on the ATPase domain of the helicase loader DnaI. Beyond designing small molecules that fit the pocket of the active site, the *in silico* approach is also able to optimise the hit compounds improving their activity and calculating their physicochemical properties, hence increasing the chance to successfully pass the subsequent preclinical stage [38].

Conclusions and outlook

As 'predators' to bacterial pathogens, virulent phages show great potential in the battle against antibiotic resistance development. Most phages can only infect a very limited range of bacterial isolates within a species. This specificity stems from the long bacteria-phage evolution and presents itself as a potentially sustainable solution using phages as precision antibacterials especially against drug-resistant *ESKAPE* pathogens. In addition to phage therapy, where phage particles are applied at infected sites, or enzyme-based antibacterials like lysins, understanding phage hijacking mechanisms can uncover numerous novel targets either for existing antibacterial drugs through new mode of action or for designing new drugs.

It is noteworthy that, this field of research is still maturing and faces challenges when expanded towards other emerging pathogens, as specific genetic tools must be available, from expression vectors and efficient transformation to the ability for genome engineering. This strategy also implies an extensive exploratory phase towards the discovery and functional understanding of individual phage-host interactions, which constitutes a long road of fundamental research before exploitation is possible. In addition, the interdisciplinary nature of the research requires techniques ranging from microbial omics analysis, functional and structural biochemistry to drug discovery related bioinformatics and chemistry, which are very challenging to integrate. However, considering the high value of these evolution-optimised interactions, compared to the randomness of current small molecule screening, this extended development line could be worth implementing.

Furthermore, recently emerging strategies exploiting bacteriophages have extended beyond their original antibacterial traits towards other alternative approaches. In addition to the direct killing of the hosts, anti-virulent agents may also be explored in phage research [46–49]. Pre-treatment with such compounds are likely to result in a reduced virulence of the pathogen, making these bacteria more susceptible to phagocyte engulfment and serum killing [46,49]. Therefore, the human immune system becomes the essential third party in the battlefield of phage and bacteria. This critical triangle thus becomes the key towards both the development of sustainable antimicrobials and understanding the biological and evolutionary dynamic equilibrium between human and microbiome.

Conflicts of interest statement

Nothing declared.

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CRediT authorship contribution statement

Xing Wan: Conceptualization, Visualization, Writing - original draft, Writing - review & editing. **Hanne Hendrix:** Conceptualization, Visualization, Writing - original draft. **Mikael Skurnik:** Conceptualization, Supervision, Writing - review & editing. **Rob Lavigne:** Conceptualization, Visualization, Supervision, Project administration, Writing - review & editing.

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